70% A and 30% B over 10 min (Rinchik et al., 1993). The solvent was evaporated from each fraction and the products were extracted with CH_2Cl_2 and characterized by ^1H and ^{13}C NMR.

Sequential absorption spectra of aqueous **3** as a function of anaerobic sonolysis at pH 7.4, 100 mM Hepes, saturating Ar, are shown in Figure 4A. The absorbance at 374 and 520 nm decreases linearly as a function of sonolysis time, and the absorbance at 316 and 420 nm increases linearly, thereby indicating the reaction is zero order in substrate concentration. The isosbestic points at 336, 390, 486 and 585 nm are in agreement with those obtained through anaerobic photolysis of CH_3-Cbl^{III}. The absorption band at 374 nm is characteristic of a Co-C bond, and its disappearance unambiguously indicates displacement of the axial carbon ligand.

Figure 4B shows the change in absorbance spectra following aerobic sonolysis of a **3** solution containing phosphate buffer. Different stable products are obtained under aerobic conditions. Because of the presence of molecular oxygen, the released product was shown by NMR to be 2-[4-(4'-[bis-(2-chloroethyl)amino]phenyl)butyroxy]ethan-1-al. ^1H NMR ($CDCl_3$, 300 MHz) 9.59 (s, 1H), 7.08 (d, 2H, J=2.9 Hz), 6.62 (d, 2H, J=2.9 Hz), 4.67 (s, 2H), 3.73-3.59 (m, 8H), 2.60 (t, 2H, J=7.5 Hz), 2.45 (t, 2H, J=7.4 Hz), 1.95 (quintet, 2H, J=7.4); ^{13}C NMR ($CDCl_3$, 75 MHz ^1H decoupled) 195.85, 173.09, 144.54, 130.43, 129.92 (2), 112.29 (2), 68.73, 53.74 (2), 40.69 (2), 33.99, 33.13, 26.79. The decrease in absorbance at 374 nm is linear with increasing sonolysis time indicating the reaction is zero order in substrate concentration.

The Co-C bond of CH_3-Cbl^{III} can be cleaved by sonolysis in aqueous solutions to produce the alkane and cob(II)alamin under anaerobic conditions or to produce the aldehyde and hydroxocob(III)alamin under aerobic conditions. Unlike photolysis and thermolysis that lead to direct Co-C bond cleavage, the predominant pathway for Co-C bond cleavage by sonolysis is through H• mediated reduction of CH_3-Cbl^{III} to the labile 19 e^- CH_3-Cbl^{II-} species followed by dissociation to the closed-shell alkane and Cbl^{II}, or through the reaction of HO• with CH_3-Cbl^{III} that leads to formation of hydroxocob(III)alamin as well as degradation of the corrin ring.

A parallel exists between the reactions of alkylcob(III)alamin under the conditions of sonolysis and pulse radiolysis, (Blackburn et al., 1972) but without the need for expensive equipment. Although the violent cavitation during sonolysis has sufficient energy to break the Co-C bond to produce the {R• •Cbl^{II}} radical pair by a dissociative pathway analogous to the photolysis of CH_3-Cbl^{III}, (Endicott and Netzel, 1979; Chagovetz and Grissom, 1993; Natarajan and Grissom, 1996), alkylcob(III)alamins are not sufficiently volatile to be found in the extreme

environment of the collapsing bubbles. Therefore, *direct* Co-C bond cleavage by sonolysis is not possible in spite of the more than 80 kcal/mol difference in bond-dissociation energy between Co-C and H-OH.

Anaerobic sonolysis of the Co-C bond is irreversible because a closed-shell alkane is formed. Under aerobic conditions, the rate of H• reaction with O_2 is on the same order of magnitude as the reaction of H• with CH_3 , thereby suggesting the closed-shell alkane, CH_4, should be one of the end products of CH_3-Cbl^{III} sonolysis (Buxton et al., 1988; Baulch et al., 1992). In contrast, Co-C bond cleavage of CH_3-Cbl^{III}, *via* anaerobic photolysis, is reversible from the $\{CH_3\bullet \bullet Cbl^{II}\}$ radical pair.

In summary, the ability to form cob(II)alamin and the closed-shell alkane without the use of chemical reductants and without the use of electrochemical, photochemical, or pulse radiolysis equipment may be a useful method to promote activation of drug-cobalamin complexes *in vivo*.

EXAMPLE 8

Materials and Methods for *in vitro* Assays of Bioconjugate Activity

Media Preparation

All media were purchased from Sigma and materials used to supplement the media were purchased from Atlanta Biologicals. The HL-60 cell culture was grown in an α-MEM media. The media was completed prior to inoculation by the addition of reagents to bring the final media concentration to 7.5% w/v sodium bicarbonate, 10% fetal calf serum, 100 üg/mL, penicillin and streptomycin, and 50 units/mL mystatin. McCoy's media, with sodium bicarbonate buffer, was used for HCT-116 cell culture. It was completed in the same manner with 8% newborn calf serum and 2% fetal calf serum. Completed media could be stored at 4°C for several weeks. The culture medium was warmed to 37°C before inoculation with cells.

Stock Culture Preparation and Maintenance

Stock cell cultures were started from ATCC cell lines. The original ATCC cell line was aliquoted in 10% DMSO and stored in liquid nitrogen. Stock cultures of 40 mL were grown and maintained in collaen-treated, sterile 75 mL culture flasks purchased from Corning. The cultures were incubated at 37°C in a 5% CO_2 environment to maintain a pH of 7.1. Humidity within the

incubator was maintained to prevent hypertonicity in the media by placing an open pan of water in the bottom of the incubator.

The concentration of cells within the stock culture was controlled and cell concentrations were estimated in several ways. The media became more purple and subsequently orange as a result of cell metabolism and metabolic byproducts that accumulated in the media. The cells were also visually observed under microscope at 40X and 100X power. Normal HCT-116 cells appeared rounded, flat, and adhered strongly to the walls of the culture flask. When the cells almost covered the bottom of the flask, the cell concentration was reduced. Normal HL-60 cells appeared round, but were well differentiated and easily suspended in the media. Changes in cell morphology were often indicative of bacterial or fungal contamination. For the accurate determination of cell concentrations, a Coulter Cell Counter™ was employed. Stock cultures were not allowed to grow to greater than 100,000 cells/mL. Both of the cell lines were observed to have a doubling time of about 24 hrs.

Assay Preparation

The assays were performed in collagen-treated, sterile 96-well plates that were purchased from Corning. HL-60 cells were grown in round-bottomed wells (Corning catalog #25850) and HCT-116 cells were grown in flat-bottomed wells (Corning catalog #25860). Cell concentrations were measured by a Coulter Cell Counter™. Cells were diluted in bulk and loaded onto the plates with 200 µL in each well. The assays were performed using approximately 25,000 HL-60 cells/well and 40,000 HCT-116 cells/well.

Since HL-60 cells grow in suspension, the cell concentration was measured and diluted directly. HCT-116 cells, however, adhere to the walls of the flask and must be suspended by treatment with trypsin. A 0.025 mg/mL trypsin solution was thawed immediately before use. The bulk culture medium was removed by aspiration and 2mL of the cold trypsin solution were added to the flask. The flask was agitated periodically to promote suspension of the cells. Care was taken to limit cell exposure to the trypsin solution to less than five minutes, since prolonged exposure will damage the cell membrane. When the cells were suspended, as observed by a microscope, 8 mL of media were added to inactivate the trypsin. The cell concentration in the resulting suspension was measured, the suspension diluted appropriately, and loaded onto the